

IMMORTAL AVIAN CELLS

The present invention relates to avian cell lines and their derivatives.

5 It is not possible to establish cell lines spontaneously from organs taken from avian species, as can be done in the case of some organs derived from mammalian species.

10 The only available cell lines to date were obtained using the transforming properties of certain avian viruses which possess oncogenic properties, such as the retroviruses of the avian leukosis group or Marek's disease virus, or certain chemical molecules such as methylcholanthrene and diethylnitrosamine.

15 For the most part, these cell lines are considerably transformed, which renders them unsuitable for multiplying vaccine viruses.

20 Authors are adopting a novel approach which consists in introducing into cells a vector which does not exhibit any oncogenic character but which is able to integrate, into these cells, a gene which is selected for its capacity to induce immortalization.

25 The first tests were carried out using vectors which integrate avian retrovirus genes such as erbA and erbB.

30 French Patent Application FR-A-2 596 770 proposes an immortalization method in which a culture of avian or mammalian cells is infected with a vector or a system which, while not being oncogenic for the said cells, is able to integrate a gene selected from v-myb, v-ets and v-erbA into these cells. The AMV, E26 and XJ12 viruses, with this latter being a virus derivative of the AEV virus in which the oncogenic v-erbB gene has been deleted, can be appropriate vectors.

35 In practice, while these tests made it possible to obtain established cell lines from cells of the haematopoietic cell line, they did not give the expected results in the case of chick embryo cells in adherent culture, such as fibroblasts or epithelial cells.

It was possible to obtain untransformed avian cell lines of the myeloblastoid type (blood cells) using the oncogene myb (International Patent Application WO91/18971).

5 In parallel, authors have proposed using the early t and T genes of the simian virus SV40 for immortalizing cells derived from different mammalian tissues (D.S. Neufeld et al., Molecular and Cellular Biology, August 1987, 2794-2802, O. Kellermann and F. Kelly,
10 Differentiation 1986, 32 : 74-81 and French Patent Application FR-A-2 649 721).

For its part, French Patent Application FR-A-2 649 721 proposes a method of conditional immortalization which, it is claimed, can be used for any cell type and
15 in any species, with the aim in this case being that of remedying the drawback of the high degree of specificity of the conventional approaches (limitation to particular species and/or to particular cell types): transformation of cells with a transforming virus (adenovirus, Epstein-
20 Barr virus, certain papovaviruses such as the SV40 virus or polyoma virus; for example, the SV40 virus is indicated as only transforming rodent cells and human cells); transfection with constructs which contain a transforming gene which is linked to a viral promoter;
25 transfection with a transforming gene which is linked to a cellular promoter. The choice of this patent application falls on a construct which combines a DNA fragment from the regulatory sequence of vimentin and a DNA fragment which encodes an immortalizing gene, which
30 construct can be the T antigen of the SV40 virus under the control of the inducible promoter of vimentin. This document never mentions the avian species.

The actual use of such viral oncogenes has never been described in the avian species, apart from the use
35 of the 12S form of the E1A protein of human adenovirus 5, which made it possible to immortalize quail epithelial cells (Guilhot et al. (1993), Oncogene 8 : 619-624).

Contrary to all expectations, the inventors succeeded in producing immortal, untransformed avian cell lines.

More generally, the inventors have found that it was possible to prepare immortal, untransformed avian cell lines which are resistant to apoptosis even from cells of avian tissues, that is to say from cells other than circulating blood cells or haematopoietic cells.

The present invention therefore relates to immortal, untransformed avian cells which are resistant to apoptosis and which derive, in particular, from avian tissues, that is to say from cells other than blood cells or haematopoietic cells, in particular fibroblasts and epithelial cells, for example from embryos.

The present invention more especially relates to an untransformed, immortal avian cell line which is selected from the group consisting of:

- cell line TDF-2A bcl-2, which is deposited in the CNCM (Collection Nationale de Cultures de Micro-organismes de l'Institut Pasteur [Pasteur Institute National Collection of Microorganism Cultures]) under reference number I-1709
- cell line TCF-4.10, which is deposited in the CNCM under reference number I-1710
- cell line TCF-4.10 bcl-2, which is deposited in the CNCM under reference number I-1711

bcl-2 denotes that the cells of the cell line functionally integrate the bcl-2 gene, which confers on them resistance to apoptosis (WO-A-93/20200, which is hereby incorporated by reference).

The invention naturally covers the cells which are derived from these cell lines. By this, it is to be understood that it is not only the cells as deposited in the CNCM under the indicated references which are covered but also the cells which constitute the progeny of these deposited cells, i.e., on the one hand, those which are obtained by simple multiplication and which may undergo mutations during these multiplications and, on the other hand, those which are obtained after

deliberate modification, which are then termed the derived cells, and, of course, also those which have undergone the two types of modification.

The invention therefore also covers the derived
5 cells which are obtained by modifications of the above cells. These modifications may consist in:

- Inserting one or more expression cassettes, each of which comprises one or more nucleotide sequences encoding a molecule of industrial relevance, with these
10 expression cassettes being able to produce this molecule following insertion into the cells of the invention. The skilled person is fully conversant with the technique. Molecules of industrial relevance which may be mentioned, in particular, are viral subunits of the
15 peptide, protein or glycoprotein type, in particular for use in a vaccine or a diagnostic reagent, protein molecules such as hormones, etc.

- Chronically infecting with a virus which is able to multiply in these cells, for virus or vaccine production
20 purposes, with or without prior modification of the sensitivity towards this virus. The infection may also not be chronic but carried out on a batch of cells which is selected for the viral multiplication.

(The modifications described below are to be understood
25 as preferably and advantageously being combined with the preceding two types of modification).

- Introducing survival or anti-apoptotic genes other than bcl-2, such as the genes which encode the human adenovirus p19E1B (Rao et al. (1992), Proc. Natl. Acad.
30 Sci. USA 89 : 7742-7746), the Epstein Barr virus LMP-1 (Gregory et al. (1991), Nature 349 : 612-614) and BHRF1 (Pearson et al. (1987), Virology 160 : 151-161), the herpes simplex virus ICP34.5 (Chou and Roizman (1992), Proc. Natl. Acad. Sci. USA 89 : 3266-3270) and the
35 baculovirus p35 (Clem et al. (1991), Science 254 : 1388-1390) proteins in order to render these cell lines more resistant to the culture conditions, in particular for maintaining confluence.

- Overexpressing genes which are involved in controlling the cell cycle using vectors which are suitable for increasing the rate of proliferation. Thus, it has been demonstrated that, in certain cases, overexpressing cyclin-encoding genes leads to the cell cycle being shortened and therefore to the rate of proliferation being increased (Rosenberg et al. (1995), *Oncogene* 10 : 1501-1509; Quelle et al. (1993), *Genes and Dev.* 7 : 1559-1571).
- Modifying the viral sensitivity spectrum of the cell lines by integrating genes which encode receptors for the viruses of interest, with a view to multiplying these viruses.
- Reference may be made to the mammalian species, where expression of the receptor for the measles virus (CD46) by murine cells, which are normally non-permissive for the virus, results in these cells becoming sensitive to this virus and being able to replicate it (Naniche et al. (1993), *J. Virol.* 67 : 6025-6032). The interest is, in particular, in rendering cells sensitive to a virus in order to produce the virus on these cells.
- Integrating oncogenes which are able to accelerate cell growth.

It is self-evident that the derived cells according to the invention may comprise one or more of the above-described modifications.

The invention also relates to a method for producing molecules of industrial relevance or viruses, which method comprises culturing the above-described cells.

The present invention is directed, in particular, towards producing molecules or viruses for creating diagnostic reagents or vaccines, or else towards producing molecules of therapeutic relevance.

The invention will now be described in more detail with the aid of embodiments which are taken by way of non-limiting examples and with reference to the attached drawing, in which:

Brief Description of the Drawings

- Figure 1 shows the structure of the vector pDAMT, which is used to prepare the cell line TDF-2A, and in which:

LTR: direct repeat sequence (long terminal repeat)

5 δ LTR: deleted LTR

MTI: murine metallothionein I promoter

SV40 T+t: SV40 early region

SV40: SV40 promoter

- Figure 2 shows the structure of the vector pphMT, which is used to prepare the cell line TCF-4.10, and in which:

LTR: direct repeat sequence (long terminal repeat)

phleo: gene for resistance to phleomycin

SV40pA: SV40 polyA

15 MTI: murine metallothionein I promoter

SV40 T+t: SV40 early region

EXAMPLE 1 = Production of the TDF-2A cell line

20 **I. Description of its origin and its characteristics**

1.1 Description of the vector employed : vector pDAMT

 It comprises the SV40 virus early region
25 (encodes the T and t antigens) (HindIII/BamHI fragment)
 (Fiers et al. (1978), Nature 273 : 113-120) under the
 control of the mouse metallothionein I promoter
 (EcoRI/BglII fragment with the BglII site being
 transformed into a HindIII site) (Durnam et al. (1980),
30 Proc. Natl. Acad. Sci. USA 77 : 6511-6515 ; Brinster et
 al. (1982), Nature 296 : 39-42).

 The EcoRI/EcoRI fragment containing this tran-
 scription unit, derived from the vector pMTSVneo (Peden
 et al. (1989), Exp. Cell. Res. 185 : 60-72), was
35 inserted into the XbaI site of the vector pDA1 (Aubert
 et al. (1991), J. Cell. Biol. 113 : 497-506). This
 latter vector is essentially derived from the genome of
 the Rous sarcoma-associated virus 2 (RAV-2) following
 modification of the 3' LTR. Thus, the U3 region of the

RAV-2 3' LTR was deleted and linked to the R and U5 regions isolated from the Rous sarcoma-associated virus 1 (RAV-1) LTR. The vector also carries a transcription unit which contains the gene for resistance to neomycin under the control of the SV40 promoter derived from the vector pSV2neo (Southern and Berg (1982), J. Mol. Appl. Genet. 1 : 327-341). See Figure 1.

1.2. Establishment of the cell line and demonstration that it is immortalized.

Cells derived from 14-day Muscovy duck embryos were transfected with vector pDAMT using the dimethyl sulphoxide (DMSO) method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4 : 1172-1174. The transfected cells are then selected by applying geneticin G418 (150 µg/ml) for 15 days. The resistant clones are then subcultured regularly at a rate of from 1 to 2 passages per week. After this 3-month period of active proliferation, the cells entered into a crisis period during which most of the cells died. After this period, which lasted approximately 2 months, several clones resumed active proliferation, suggesting that they had been immortalized.

The TDF-2A cell line is thus derived from 2 cultures.

It was studied in more depth.

The TDF-2A cells achieved 200 passages, that is approximately 460 generations, and were thus maintained continuously in culture for more than 600 days. By comparison, control cells, which are not expressing the SV40 virus early region, cannot be maintained in culture for more than 20 passages.

1.3. Proliferation characteristics.

The immortalized cells are cultured at 38°C, in a roller bottle, in a medium containing 6% 10 × HAM F-10, 4% 10 × 199 HANKS, from 2.95% to 4% tryptose broth phosphate, from 5.6% to 2.5% sodium bicarbonate, 0.1% 100 × vitamin BME, 3% foetal calf serum, from 5% to 1% kanamycin and from 0.5% to 1% vancomycin.

Under these conditions, their rate of doubling is once every 24 hours.

1.4. Expression of the T antigen.

It was verified, by means of indirect immuno-
5 fluorescence or indirect immunophosphatase using an antibody which is specific for the T antigen (Pab F01 : Santa Cruz Biotechnology ref. sc147), that all the cells express the T antigen in their nucleus, indicating that they have all integrated the vector.

10 This integration was additionally demonstrated by means of Southern blotting. The genomic DNA of the immortalized fibroblasts was digested with the restriction enzymes XbaI and BstXI. Hybridization with a probe which was specific for the T antigen (1018 bp NdeI/NdeI
15 fragment) verified that the transcription unit, which expressed the immortalizing gene and which was inserted into the TDF-2A cells, had not undergone any major rearrangements. This was indicated by the fact that the sizes of the hybridization fragments obtained were in
20 accordance with the expected sizes.

1.5. Absence of tumorigenic capacity.

The immortalized cells do not exhibit any tumorigenic capacity. They are incapable of forming colonies in semi-solid medium or of forming tumours on
25 hen or duck egg chorioallantoic membrane. They are also incapable of forming tumours on nude mice, and on one-day old SPF (pathogen-free) ducklings and chicks.

1.6. Karyotype.

The karyotype of the TDF-2A cells was studied at
30 the 114th and 135th passages. This verified that the cells were indeed of avian origin, with the microchromosomes characteristic of this species being present. Furthermore, the chromosomes which were observed are representative of the chromosomes which are
35 encountered in primary duck embryo cells, thereby confirming the origin of the cell line.

II. Properties.

The TDF-2A cells exhibit, in particular, a sensitivity to the duck-specific viruses, such as

adenovirus, parvovirus and reovirus, which are customarily replicated on primary duck embryo cells. These viruses can therefore be produced on this cell line.

5 **EXAMPLE 2 : Characterization of the TDF-2A cell line by identifying the integration sites.**

The genomic DNA of the TDF-2A cells, which was prepared from cells derived from the 114th and 135th passages, was digested with the restriction enzymes
10 BglII and KpnI. The DNA, which had been treated in this way, was then subjected to gel electrophoresis, followed by transfer to a nylon membrane; it was then hybridized with a probe which was specific for the T antigen (1018 bp NdeI/NdeI fragment). For example, digestion with
15 BglII gives rise to two hybridization bands of large size (approximately 15 and 23 kb), suggesting the existence of two integration sites. Digestion with KpnI gives rise to one major band of large size (approximately 20 kb) and to at least one minor band,
20 thereby confirming the existence of at least two integration sites.

EXAMPLE 3 : Production of the TCF-4.10 cell line

1. Description of its origin and its characteristics
25

1.1. Description of the vector employed: vector pphMT

It comprises the SV40 virus early region (encodes the T and t antigens) (HindIII/BamHI fragment)
30 (Fiers et al. (1978), Nature 273: 113-120) under the control of the mouse metallothionein I promoter (EcoRI/BglII fragment with the BglII site being transformed into a HindIII site) (Durnam et al. (1980), Proc. Natl. Acad. Sci. USA 77: 6511-6515; Brinster
35 et al. (1982), Nature 296: 39-42).

The EcoRI/EcoRI fragment containing this transcription unit, derived from the vector pMTSVneo (Peden et al. (1989), Exp. Cell. Res. 185: 60-72), was inserted into the EcoRI site of the vector pUT507

(marketed by CAYLA-FRANCE), being located 3' of the region for expressing the gene for resistance to phleomycin (Figure 2). The structure of vector pUT507 is described in Mulsant et al. (1988), Somatic Cell and
5 Molecular genetics 14: 243-252.

1.2. Establishment of the cell line and demonstration that it is immortalized.

Fibroblasts derived from chick embryos were transfected with vector pphMT using the dimethyl
10 sulphoxide (DMSO) method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4: 1172-1174. The transfected cells are then selected by progressively applying (from 10 µg/ml to 50 µg/ml) phleomycin for 15 days. The resistant clones are then subcultured
15 regularly at a rate of from 1 to 2 passages per week. After a period of active proliferation of approximately 2 months, the cells entered into a crisis period when cell growth is very weak and during which there is a very high rate of mortality. After a period which
20 lasted from 3 to 4 months, a few cells of the TCF-4.10 clone resumed active proliferation, suggesting that they had been immortalized.

The TCF-4.10 cells thus achieved 200 passages in culture, that is approximately 400 generations, and
25 were maintained in culture for 3 years. By comparison, control fibroblasts, which are not expressing the SV40 virus early region, cannot be maintained in culture for more than 20 to 30 passages.

1.3. Proliferation characteristics.

30 The immortalized fibroblasts are cultured at 38°C in a medium containing 6% 10 × HAM F-10, 4% 10 × 199 HANKS, from 2.95% to 4% tryptose broth phosphate, from 5.6% to 2.5% sodium bicarbonate, 0.1% 100 × vitamin BME, 3% foetal calf serum, from 5% to 1%
35 kanamycin and from 0.5% to 1% vancomycin. Under these conditions, their rate of doubling is 0.7 times per 24 hours.

2.2. Expression of the T antigen.

It was verified, by means of indirect immuno-
fluorescence or indirect immunophosphatase using an
antibody which is specific for the T antigen (Pab 101:
5 Santa Cruz Biotechnology ref. sc147), that all the
cells express the T antigen in their nucleus, indicat-
ing that they have all integrated the vector.

2.3. Absence of tumorigenic capacity

The immortalized fibroblasts do not exhibit any
10 tumorigenic capacity. They are incapable of forming
tumours on hen or duck egg chorioallantoic membrane.

3. Properties.

The TCF-4.10 cells exhibit, in particular, a
sensitivity to avian viruses. Viruses which may, in
15 particular, be mentioned are the avian poxviruses, such
as canarypox or fowlpox, or else the Marek's disease
viruses (1, 2 and 3(HVT) serotypes) or the Gumboro
disease virus. These viruses can therefore be produced
on this cell line.

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EXAMPLE 4: Multiplication of canarypox on TCF-4.10 cells.

The TCF-4.10 cells are seeded in a roller
bottle. The canarypox is inoculated onto an established
25 lawn. When the cytopathic effect engendered by the
virus has become generalized, harvesting is carried out
by shaking so as to detach the cell lawn. The harvested
mixture therefore consists of the cell lawn and the
culture supernatant. The whole is homogenized by
30 treating with an Ultraturrax for 1 min at 13,500 rpm
(T25-type IKA appliance).

The infectious viral titre is determined by
means of a micromethod carried out on a 96-well plate.
The virus dilutions are inoculated onto a lawn prepared
35 from secondary chick embryo cells. Each viral dilution
is inoculated onto 6 wells. The plates are incubated in
a CO₂ incubator for 8 days. The presence of the virus
in the wells is checked by observing the characteristic
cytopathic effect (CPE) under the microscope. The

infectious titre is calculated by the KARBBER method and is expressed by the logarithm of the inverse of the viral dilution which gives 50% CPE [titre = $d+r/Nx(n+N/2)$], where d is the dilution expressed in logs when all the wells are positive, r is the dilution ratio, N is the number of wells per dilution and n is the number of positive wells between 0 and 100%.

Results: The viral titres obtained are equivalent to those obtained on primary duck embryo cells.

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EXAMPLE 5: Integration of the bcl-2 gene

A vector which permits expression of the bcl-2 gene under the control of the CMV (human cytomegalovirus) promoter is transfected into the TDF-2A and TCF-4.10 cells using conventional transfection methods (DMSO method described by Kawai and Nishizawa (1984), Mol. Cell. Biol. 4: 1172-1174 or lipofectamine method in accordance with the supplier's (GIBCO-BRL) recommendations).

After the transfected cells have been selected, expression of the Bcl-2 protein is detected by Western blotting.

The cells which express the Bcl-2 protein are then tested for their ability to survive under culture conditions in which an apoptosis process is observed (maintenance of the cells at confluence).

Thus, in the case of the TDF-2A bcl-2 cells, the apoptosis process engendered by the cells arriving at confluence is deferred by from 3 to 4 days as compared with the TDF-2A cells. An increase in cell density at confluence is observed in the TCF-4.10 bcl-2 cells as compared with the TCF-4.10 cells.